Separation of vascular cell walls from cortical cell walls of plant roots

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Summary. The cortex was physically separated from the stele of corn roots. The isolated walls from cortical cells were less dense than the walls isolated from stelar cells. The cell walls from each tissue were centrifuged on a step gradient composed of 50 and 60% sucrose for 5 min at 900 **g**. After the short centrifugation time, the cortical cell walls banded at the 50/60% interface while the vascular tissue walls pelleted through 60% sucrose. An aliquot of vascular cell walls was then marked with cytochrome *c*. The marked cell walls were mixed with cortical cell walls and centrifuged on a 50/60% sucrose gradient and after 5 min, the vascular tissue walls were cleanly separated from the cortical cell walls. The experiment was repeated without prior separation of tissue types with another corn variety, carrot roots grown in culture, and pea roots. A clean separation of cell wall types was achieved after homogenization of intact roots in liquid nitrogen, extrusion from a nitrogen bomb, and centrifugation in sucrose gradients.

Keywords: Cortex; Microscopy; Purified cell walls; Sucrose gradients; Vascular cylinders.

Introduction

Composition analyses of primary cell walls from intact tissue has been confounded by the presence of vascular tissue cell walls. Unlike cell suspensions, where a fairly good homogenous population of cells with primary cell walls can be found, intact tissue always has vascular tissue present. To study structural and functional differences between cortical cell walls and vascular cell walls, a method to separate the wall types is desired. Although the cortical cylinder can be readily separated from the stele (vascular tissue) of some plant roots (Hall et al. 1971, Libbenga et al. 1973), in most plant tissues it is impractical to achieve this physical separation.

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In this study, corn roots were chosen because the cortex could easily be separated from the stele (Leonard et al. 1975). By working with cell walls from both types of tissue, appropriate conditions for their separation could be determined. Sucrose gradients have been used to purify cell walls (Nakagawa et al. 1971, Nagahashi et al. 1985), and in this study, the use of sucrose gradients was extended to separate wall types. The method developed was then tested by homogenizing intact roots, without prior separation of tissue types, to determine whether cortical cell walls and vascular cell walls could be separated from a mixture.

Materials and methods

Plant material

Corn seeds (*Zea mays* L., WF9 × MO17 or FRB73) were germinated and harvested after three days growth as previously described (Nagahashi and Seibles 1986). Alaskan peas (early variety) were soaked in water and germinated in glass trays filled with vermiculite. The seedlings were harvested after 5 days growth and the primary root was excised. Carrot root cultures were grown on a gellan support medium with added nutrients (Bécard and Piché 1991). Cultured roots were harvested by solubilizing the gellan after the primary roots had developed substantial secondary root growth (Nagahashi et al. 1993).

Cell wall isolation procedures

The tips (1.0 cm) of primary corn roots were excised and the cortex of the remaining root was physically pulled from the stele as described previously (Leonard et al. 1975). Cortical cylinders were pulverized in liquid nitrogen, and suspended in homogenization medium containing 0.3 M sucrose plus 5 mM sodium metabisulfite in 0.1 M HEPES-MES buffer, pH 7.8. The suspension was transferred to a 40 ml capacity Parr nitrogen bomb at 4 °C for 15 min at 1800 psi. After extrusion to atmospheric pressure, the suspension was treated under vacuum for 5 min at 4 °C to degas the preparation and finally

sonicated as previously described (Nagahashi and Seibles 1986). Isolated stele was treated exactly the same and kept separated from the cortical cell walls.

Sucrose density gradient separation of vascular cell walls from cortical cell walls

The separated cell wall types were used to determine which density gradient centrifugation conditions could be used to separate a mixture of cell wall types. A step gradient composed of 4 ml of 50% (w/w) sucrose and 9 ml of 60% (w/w) sucrose was overlaid with 1 to 2 ml of cell wall mixture and centrifuged at room temperature at 900 **g** for various periods of time. To monitor the stelar cell walls in a mixture, they were stained with cytochrome *c* (cyt *c*) which allowed them to be distinguished from cortical cell walls. A stock solution of 4 mg/ml of cyt *c* was diluted 1 : 1 with 1 ml of cell wall suspension. For monitoring purposes, either cell wall type can be stained with cyt *c*.

Light and electron microscopy

For light microscopy, cell walls were stained with methylene blue and wet mounts were viewed and photographed with an Olympus SZH light microscope system. For electron microscopy, all cell wall fractions were fixed for 1 h at room temperature in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.0. The walls were then post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol or acetone and embedded in EM Bed 812 (EMS, Fort Washington, PA) or Spurr's epoxy resin. Thin sections were post-stained with lead citrate-uranyl acetate and observed with a Philips CM 12 scanning-transmission electron microscope. For scanning electron microscopy, wall samples were air dried on glass cover slips. Cover slips were first rinsed with ethanol. Dried wall samples were coated with gold using a Denton DV-502 vacuum evaporator. The edge of the glass cover slip was painted with colloidal silver, samples were viewed with a JSM-840A SEM, and micrographs were taken with Polaroid #55 film. (Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.)

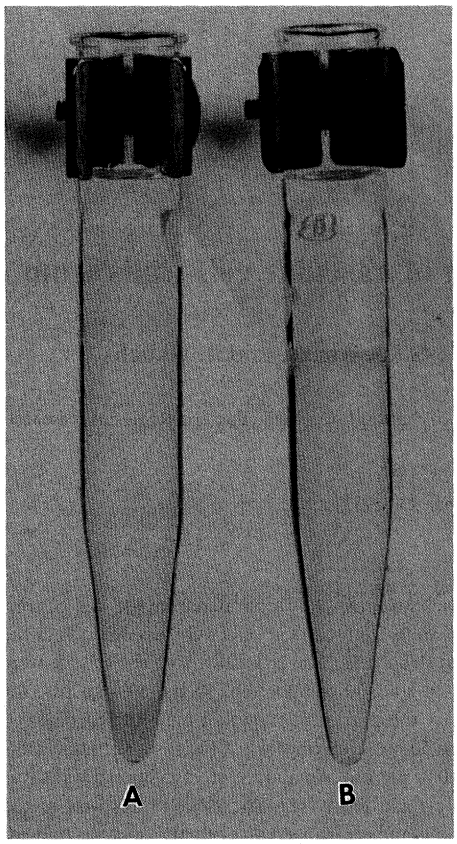


Fig. 1 A, B. Sucrose gradient centrifugation of cell wall types from corn roots. The root cortex was physically separated from the stele. **A** Vascular cell walls isolated from corn root stele were centrifuged in a step sucrose gradient (50/60%, w/w) for 5 min at 900 **g** at room temperature. The walls readily pelleted through the gradient. **B** Isolated cortical cell walls were centrifuged as in **A**, and banded at the 50/60% sucrose interface

Results

Centrifugation of isolated cortical cell walls and vascular cell walls in sucrose gradients

To determine if cell walls from the cortical region of roots had different sedimentation properties than the vascular tissue cell walls, it was first necessary to work with a root system where the cortex could be physically separated from the stele. The WF9 hybrid corn provides a nice system for separating these two tissue types. Other corn varieties (such as FRB73) are considerably more difficult to process (Nagahashi et al. 1990) and were avoided in the first experiments. Initial sedimentation observations in aqueous solution with isolated vascular walls indicated that these walls were inherently more dense than cortical cell walls.

Cell walls isolated from cortex and stele were centrifuged in various sucrose solutions under different centrifugal forces. High centrifugal forces (greater than 10,000 **g**) were of no practical value since vascular and cortical walls rapidly pelleted under these conditions. The vascular walls readily sedimented in all sucrose solutions tried and readily pelleted through 60% sucrose under low centrifugal forces. Figure 1 A shows vascular cell walls readily pelleting through 60% sucrose after 5 min at 900 **g**. On the other hand, cortical cell walls were trapped at the 50/60% sucrose interface under the same centrifugation conditions (Fig. 1 B).

The results above were confirmed by mixing the cell wall types and centrifuging in the same gradient (Fig. 2). To monitor the vascular cell walls in the mixture, these cell walls were first treated with a saturating amount of cyt *c*. The unbound cyt *c* was removed by repeated washing of the cell walls with water. The red vascular cell walls were then mixed with unstained cortical walls, overlaid on a step gradient, and centrifuged

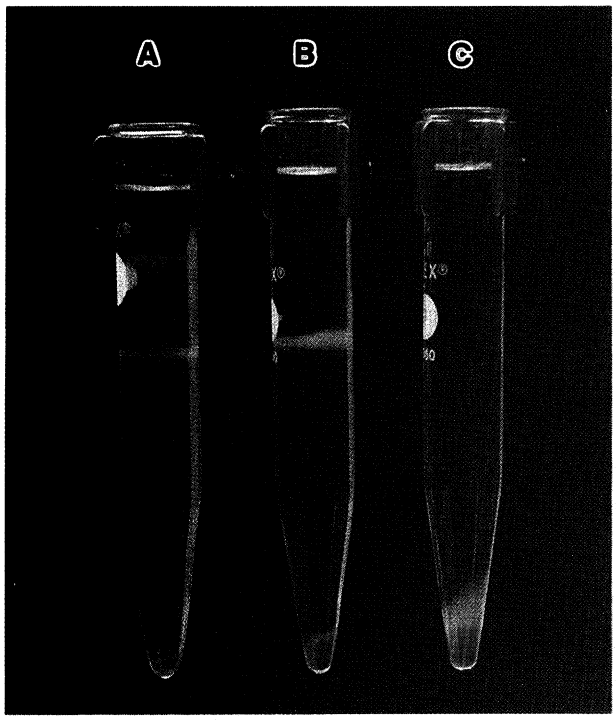


Fig. 2 A–C. A time course for sucrose gradient centrifugation separation of corn root vascular cell walls from cortical cell wall. The root cortex was physically separated from the stele and cell walls were isolated from each tissue type (see text for details). The vascular cell walls were stained with cyt *c* and could be distinguished from cortical cell walls in a mixture. Gradients were identical to those in Fig. 1 and were centrifuged at room temperature. **A** Separation after 2.5 min at 900 **g**. **B** Separation after 5 min at 900 **g**. **C** Results after 10 min at 900 **g**

for 2.5, 5, and 10 min at room temperature at 900 **g**. Figure 2 clearly showed that centrifugation time was critical for separating a mixture of cell wall types. After 2.5 min, some vascular walls were still present at the 50/60% sucrose interface and the cortical walls were found at both the top of the gradient and at the interface (Fig. 2 A). The cortical cell walls were trapped at the 50/60% interface and were completely separated from vascular cell walls after 5 min of centrifugation (Fig. 2 B). However, after 10 min of centrifugation, the cortical cell walls pelleted on top of the vascular walls (Fig. 2 C).

Light and electron microscopy of isolated cell wall preparations

Figure 3 A shows a low magnification photograph of the cortical cell walls collected at the 50/60% sucrose interface. Figure 3 B shows the vascular cell walls which pelleted through the gradient. At the light microscope level, the cortical cell walls could be readily distinguished from the vascular cell walls by their distinct differences in morphology. Clumps of cortical cell walls were amorphous and walls from individual cells were easy to observe. Vascular tissue walls retained a distinct rod shape with periodic striations. Another important point is the size of the tissue-wall fragments for both tissue types. These cell wall pieces are considerably larger than wall fragments achieved by high shear techniques such as the polytron and ball milling processes. Ultrastructural examination of the cortical cell walls showed an extremely clean cell wall preparation (Fig. 4 A). No membrane or cytoplasmic contaminants were evident. The vascular cell walls, however, did contain membrane fragments and vesicles (Fig. 4 B) even though the wall type was homogenous (Fig. 3 B).

Separation of cell wall types from intact root systems

The above experiments indicated that the cortex did not have to be separated from the stele in order to separate vascular cell walls from cortical cell walls. Because root tips were excised before cortex was separated from the stele, it was important to first determine how the cell walls of root tip material distributed in step gradients before using whole roots. The cell walls from root tip tissue remained at the 50/60% sucrose interface after centrifugation at 900 **g** for 5 min (data not shown). This was not surprising since vascular tissue is not developed in this region of the root.

Cell walls were then isolated from intact primary roots of a corn variety (FRB73) in which it was difficult to separate the cortex from the stele (Nagahashi et al. 1990). Whole intact roots were homogenized in liquid nitrogen, extruded from a nitrogen bomb, degassed, sonicated and centrifuged on the 50 to 60% step gradient. The cortical cell walls were readily separated from the vascular tissue walls by sucrose gradient centrifugation confirming our initial observations. The cortical cell walls and vascular cell walls were virtually identical to those in Fig. 3 A and B. This finding indicated that the technique could be applied to roots or other plant tissues where the cortex and stele cannot easily be separated.

Separation and purification of cortical and vascular cell walls derived from intact pea roots (data not shown) and cultured carrot roots were also achieved by this procedure. Figure 5 A shows a low magnification light micrograph of a crude carrot cell wall preparation before sucrose gradient centrifugation. To enhance the difference between vascular cell walls and

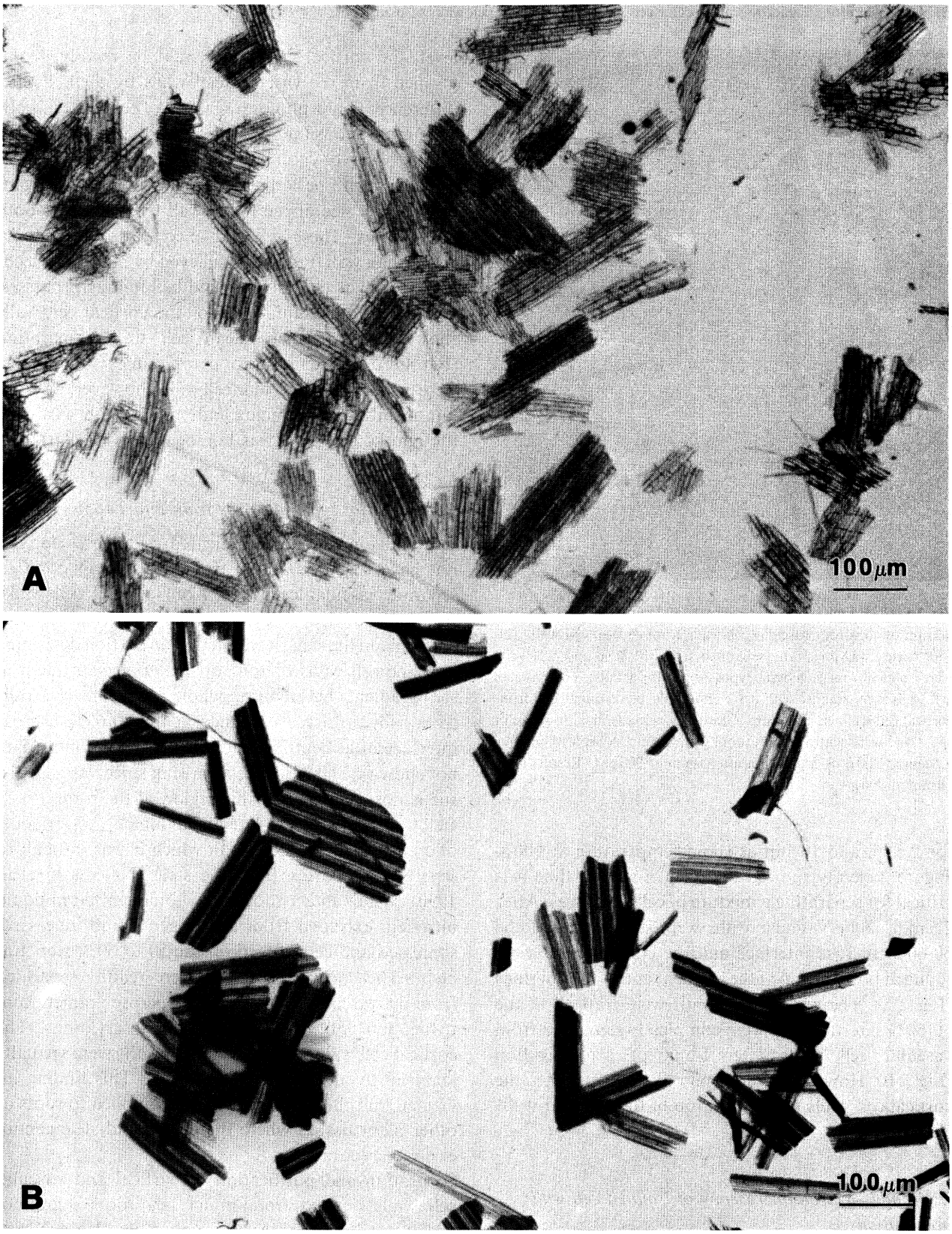


Fig. 3 A, B. Light micrographs of corn root cortical cell walls and vascular cell walls separated by sucrose gradient centrifugation and stained with methylene blue. **A** Cortical cell walls collected from the 50/60% sucrose gradient interface. **B** Stelar cell walls which pelleted through 60% sucrose

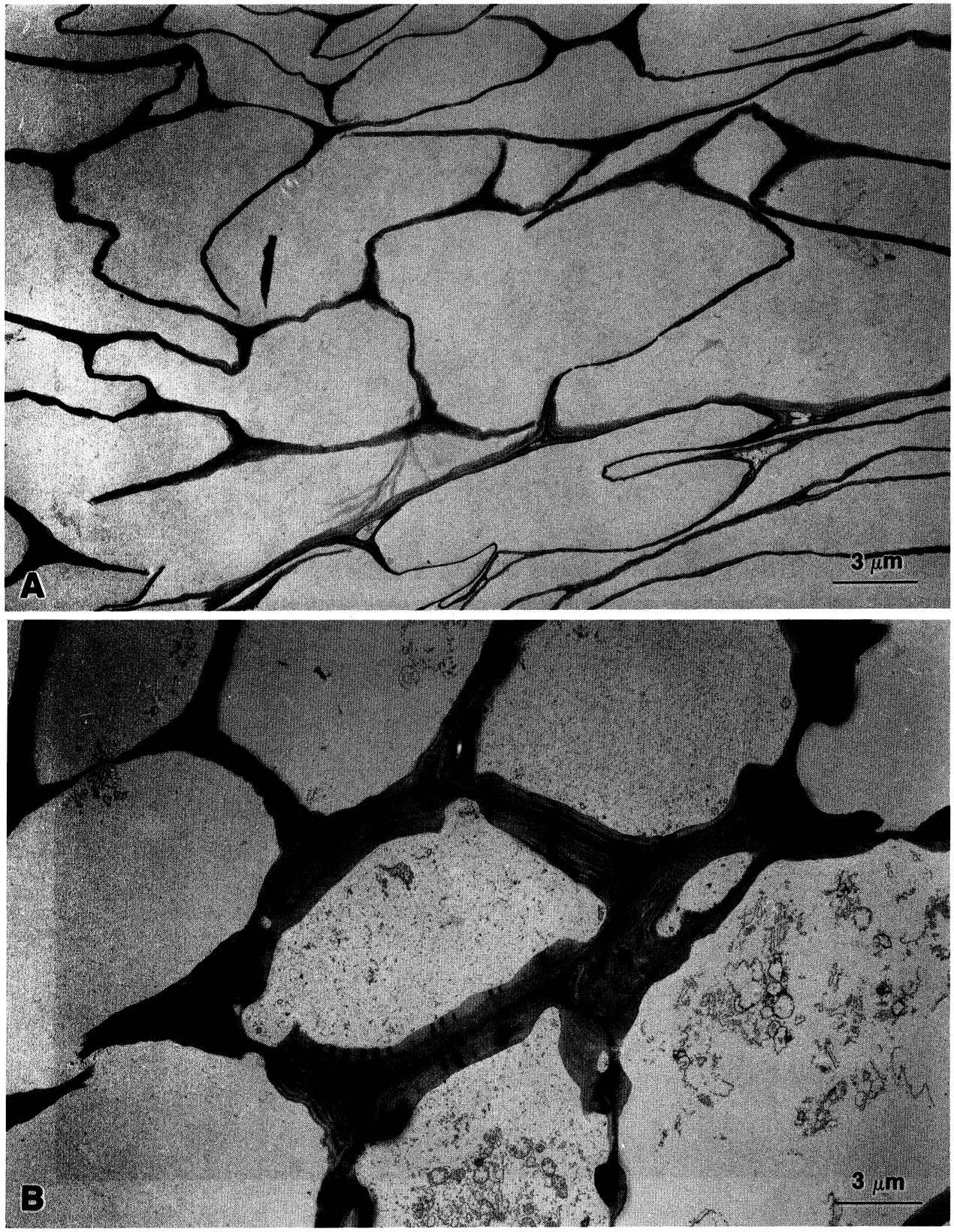
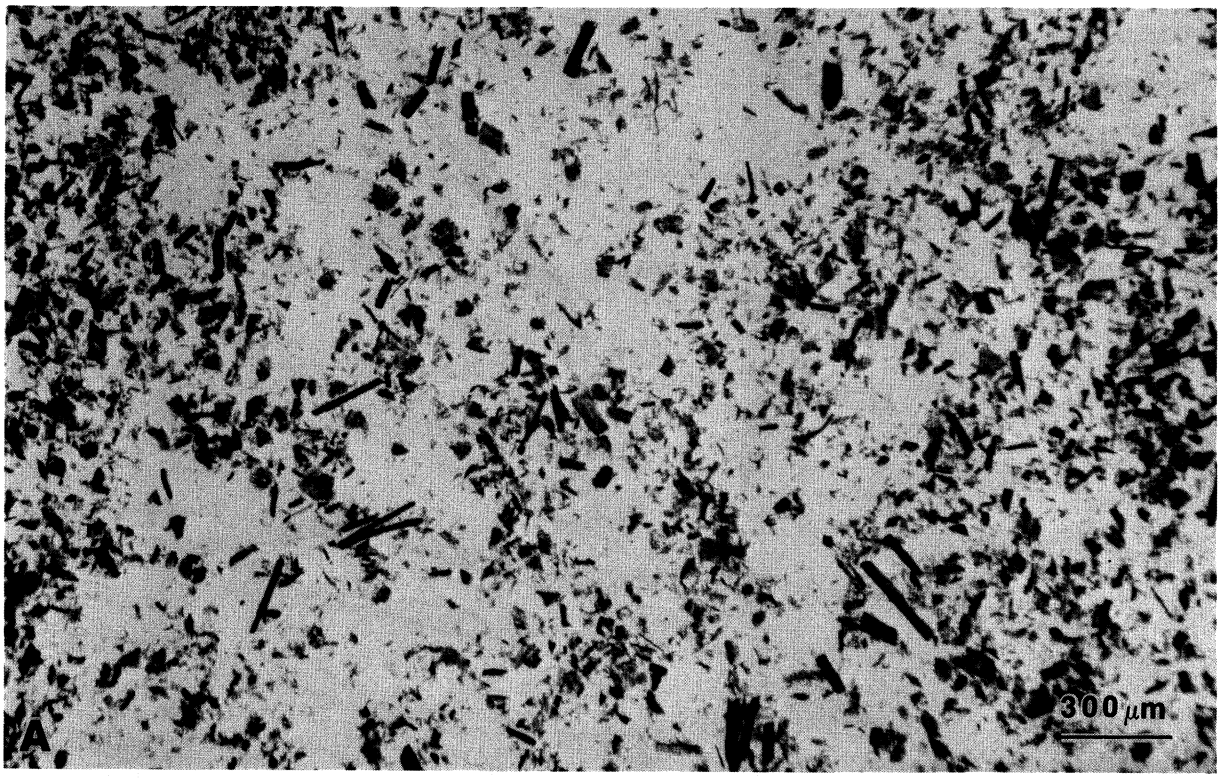


Fig. 4 A, B. Transmission electron micrographs of corn root cortical cell walls and vascular cell walls separated by sucrose gradient centrifugation. **A** Representative sample of cortical cell walls. **B** Representative sample of vascular cell wall material


A
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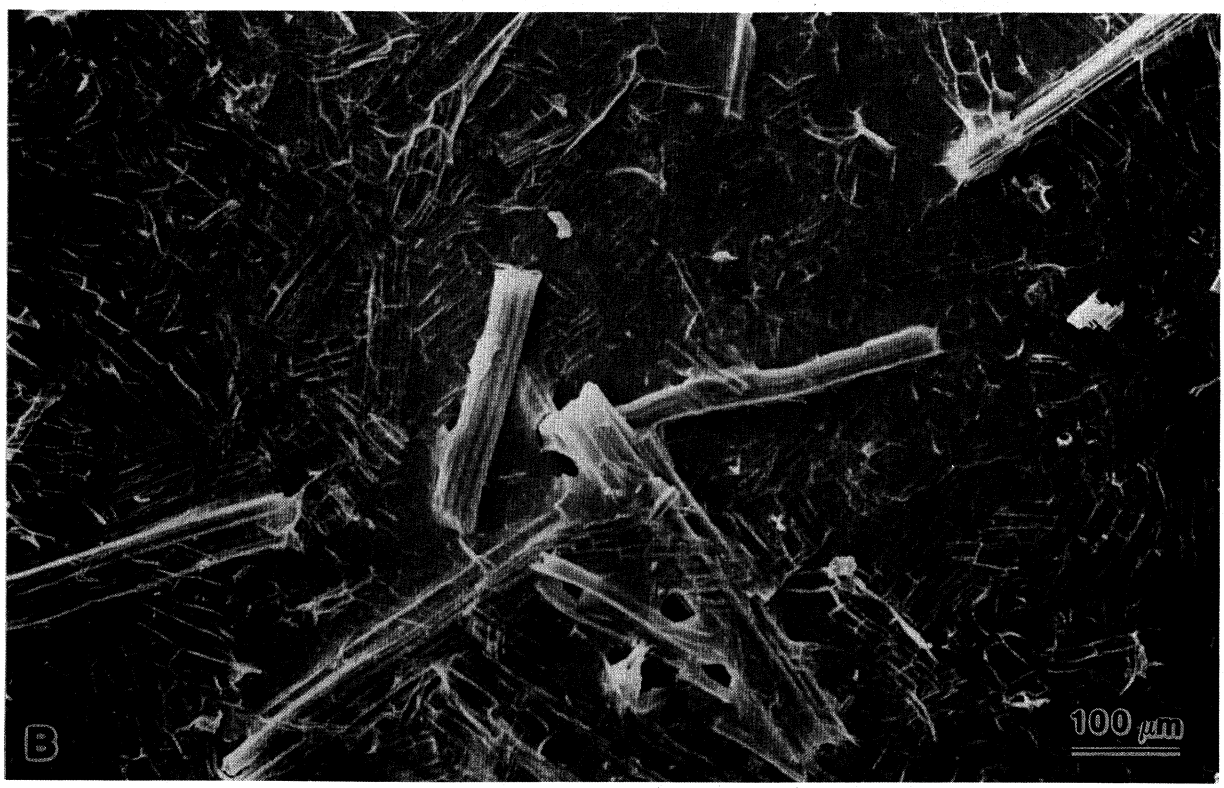
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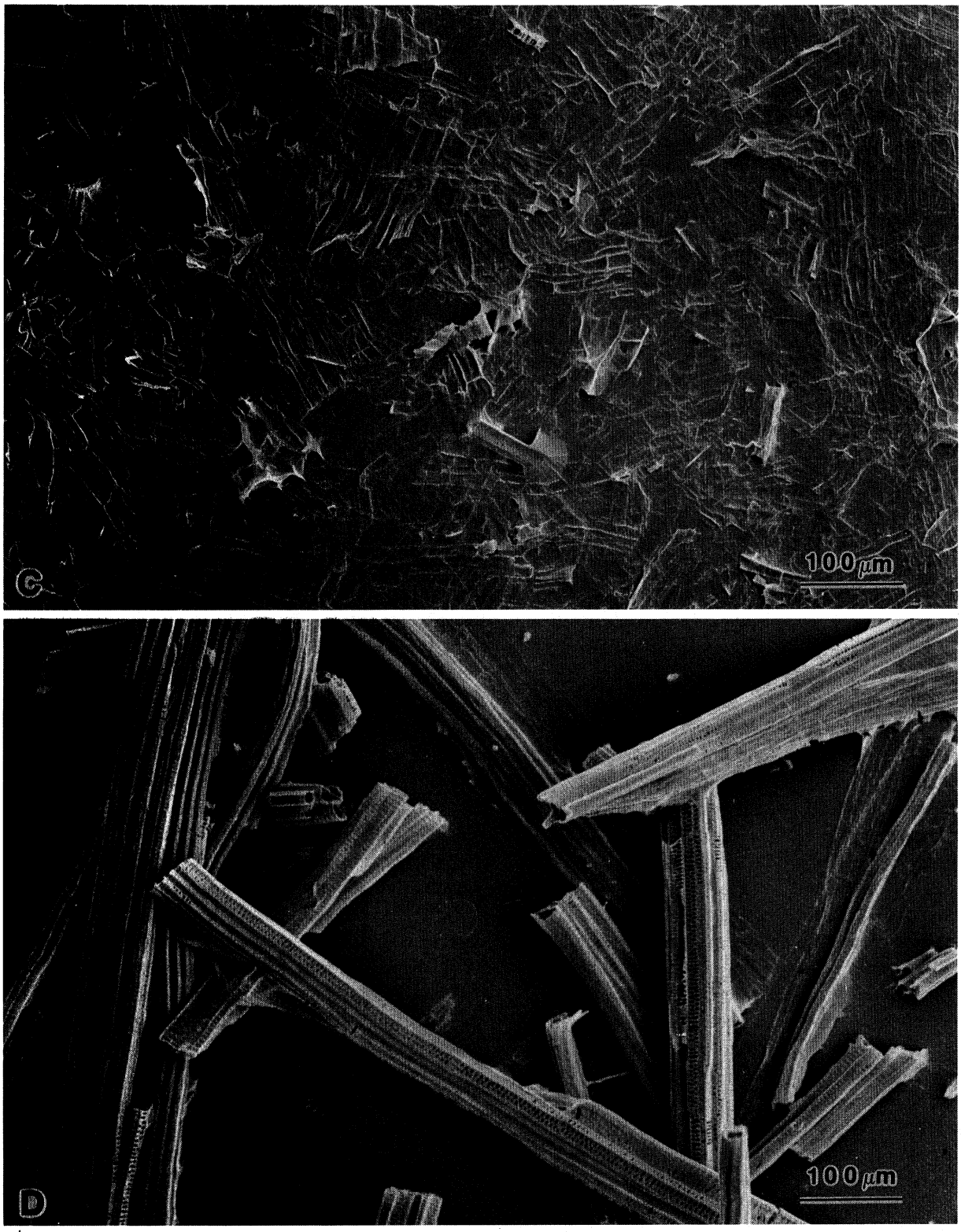

Fig. 5 A–D. Low magnification of light micrographs and scanning electron micrographs of cell wall preparations from cultured carrot roots. For SEM cell wall fractions were dried down on glass cover slips and coated with gold. Cortical walls dried flat and individual cells were easy to identify. Vascular cylinders retained their shape and could be distinguished from cortical cell walls in a mixture. In the crude homogenate, the amount of vascular cell walls was considerably less than cortical cells walls. **A** Crude cell wall preparation as viewed with a light microscope. **B** SEM micrograph of the crude cell wall preparation. **C** Cortical cell walls from the 50/60% interface of a step gradient. **D** Vascular cell walls which pelleted through the gradient

cortical cell walls, the crude cell wall fraction was air dried on a glass cover slip and viewed with an SEM (Fig. 5 B). Figure 5 C and D shows the purified cortical cell walls and vascular cell walls, respectively, as viewed with SEM. The purified fractions were washed in deionized-distilled water several times to remove the sucrose. The cortical cell walls dried down flat while the vascular cylinders retained their shape. This readily allowed to distinguish vascular wall from cortical walls in a mixture.

Discussion

The cell walls isolated from vascular tissue were more dense than the cell walls isolated from cortical cells. This difference was used to separate mixtures of these wall types by performing rate sedimentation centrifugation in sucrose gradients. It should be stressed that the separation is a rate sedimentation and not an equilibrium density gradient separation. Separation can only be achieved by centrifugation under low centrifugal force for only a few minutes. If the gradients were centrifuged at 900 **g** for 10 min or longer, the cortical cell walls pelleted on top of the vascular walls.

It should also be stated that in Figs. 1 and 2, equal proportions of vascular cell walls were mixed with cortical cell walls. This was done in order for the pellets to be clearly photographed. In reality, the vascular walls represent a much smaller portion of the total cell wall homogenate (as seen in Fig. 5 A and B) and the amount of pelleted material (vascular walls) will be considerably less than the cortical cell walls which band at the interface. When optimizing conditions for density gradient separation, one should only observe small pellets from vascular tissue.

Altough the ability to separate cortex from the stele from certain plant tissues has been available for years (Leonard et al. 1975 and references therein), the technique has not been used to study differences in vascular walls compared to cortical cell walls. The method developed in this report has a wider application for those roots or shoots in which it is not easy to physically separate cortex from the stele. We have also achieved a clean separation of cortical cell walls from vascular cell walls of carrot roots. This was significant because unlike primary roots of corn or peas, which were uniform in size, carrot roots had primary and secondary roots of different lengths and diameters. In this case, the cortical cell wall fraction contained a small amount of small pieces of vascular cell walls (Fig. 5 C) but the contamination was minimal. Likewise the vascular cell walls showed minimal contamination by cortical cell walls (Fig. 5 D).

Although the vascular cell walls could be readily separated from the cortical cell walls, the cell walls from the vascular tissue could not be purified from cellular contaminants as cleanly as walls isolated from cortical cells. This was probably due to the presence of secondary walls in vascular tissue which do not readily break under the pressure used during extrusion from the nitrogen bomb. This observation was confirmed by noting that pea leaf tissue extruded from the nitrogen bomb still retained intact guard cells (pictures not shown). However, by solvent extracting (chloroform : methanol, 2 : 1) the isolated vascular cylinders, all membrane contaminants could be rapidly removed from the vascular tissue walls if desired.

Perhaps the key to success of this technique is the pulverizing of root tissue in liquid nitrogen to create somewhat uniform tissue fragments. The crushing and pulverizing by this technique provides small pieces of tissue rather than small fragments of cell walls. Also the pulverizing probably mechanically shears the cortex from the stele and also allows fragmentation of the stele. Without liquid nitrogen, the mortar and pestle homogenization cannot efficiently break up the stele into small pieces. The extrusion from the nitrogen bomb helps break the remaining intact cells.

The technique developed here is ideal for experiments using small amounts of root tissue (1 g to 10 g fresh weight) where efficient recovery of cell walls is essential. The sucrose gradients rapidly remove cytosolic components and membranes from cortical cell walls because these contaminants cannot penetrate the 50% sucrose step at the top of the gradient. This provides an alternate method for removing extraneous cytoplasmic contaminants instead of filter-trapping (Nagahashi et al. 1986) or multiple washing by differential centrifugation (Li et al. 1983).

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